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## Effect of Breed on Plasma Endothelin-1 Concentration, Plasma Renin Activity, and Serum Cortisol Concentration in Healthy Dogs

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**Background:** There are breed differences in several blood variables in healthy dogs.

**Objective:** Investigate breed variation in plasma endothelin-1 (ET-1) concentration, plasma renin activity, and serum cortisol concentration.

**Animals:** Five-hundred and thirty-one healthy dogs of 9 breeds examined at 5 centers (2–4 breeds/center).

**Methods:** Prospective observational study. Circulating concentrations of ET-1 and cortisol, and renin activity, were measured using commercially available assays. Absence of organ-related or systemic disease was ensured by thorough clinical investigations, including blood pressure measurement, echocardiography, ECG, blood and urine analysis.

**Results:** Median ET-1 concentration was 1.29 (interquartile range [IQR], 0.97–1.82) pg/mL, median cortisol concentration 46.0 (IQR, 29.0–80.8) nmol/L, and median renin activity 0.73 (IQR, 0.48–1.10) ng/mL/h in all dogs. Overall, breed differences were found in ET-1 and cortisol concentrations, and renin activity ( $P < .0001$  for all). Pair-wise comparisons between breeds differed in 67% of comparisons for ET-1, 22% for cortisol, and 19% for renin activity, respectively. Within centers, breed differences were found at 5/5 centers for ET-1, 4/5 centers for cortisol, and 2/5 centers for renin activity. Newfoundlands had highest median ET-1 concentration, 3 times higher than Cavalier King Charles Spaniels, Doberman Pinschers, and Dachshunds. Median renin activity was highest in Dachshunds, twice the median value in Newfoundlands and Boxers. Median cortisol concentration was highest in Finnish Lapphunds, almost 3 times higher than in Boxers.

**Conclusions and Clinical Importance:** Breed variation might be important to take into consideration when interpreting test results in clinical studies.

**Key words:** Biomarker; Breed variation; Canine; Vasoactive.

Regulation of blood pressure is complex involving brain, kidneys, cardiovascular system, and vasoactive peptides. Decrease in blood pressure activates the sympathetic nervous system, the renin-angiotensin-aldosterone system (RAAS), and vasopressin, leading to vasoconstriction.<sup>1</sup> Angiotensin II can stimulate the release of endothelin-1 (ET-1), a potent vasoconstrictor, from vascular endothelium, and ET-1 can in turn stimulate the release of norepinephrine, angiotensin II, and vasopressin.<sup>2,3</sup> The RAAS, together with vasopressin, also stimulates sodium and fluid retention.<sup>1</sup> This might be counteracted by the natriuretic peptides, which

### Abbreviations:

ANP	atrial natriuretic peptide
CKCS	Cavalier King Charles Spaniel
CVs	coefficients of variation
ELFA	enzyme-linked fluorescent assay
ET-1	endothelin-1
IQR	interquartile range
NT-proBNP	N-terminal pro-B-type natriuretic peptide
RAAS	renin-angiotensin-aldosterone system
RIA	radioimmunoassay

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Planning of the study started in 2007. The study protocol was finalized in 2008. There was a follow-up meeting in 2010. The current article was finalized and approved by the investigators in August 2015. The publication committee consisted of Katja Höglund, Anne-Sophie Lequarré, Ingrid Ljungvall, and Jens Häggström.

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stimulate natriuresis, diuresis, and vasodilation.<sup>1</sup> The systems are also activated in development of cardiac and renal disease and several of these substances have been suggested as biomarkers of disease in dogs.<sup>4-6</sup> However, many physiologic and pathologic factors might influence neuroendocrine concentrations,<sup>5,7,8</sup> making interpretation of test results difficult. Dog breed is one potential such factor.<sup>9</sup>

In an European research project, LUPA, over 500 dogs of 9 breeds were examined at five centers looking for genetic determinants of variation in blood pressure, neuroendocrine, and metabolic variables.<sup>10</sup> Simultaneously, breed differences in physiological variables were investigated. Results have shown substantial breed differences in plasma concentrations of the natriuretic peptides pro-ANP 31-67 and NT-proBNP,<sup>11</sup> prompting cautiousness when using them as biomarkers for heart disease. When investigating a subpopulation of the dogs, we also found breed differences in blood pressure and heart rate in consistency with other studies,<sup>12-14</sup> and in urinary epinephrine and norepinephrine concentrations.<sup>15</sup>

It has been hypothesized that plasma ET-1 in horses could be increased in stress conditions and there are breed differences in plasma ET-1 and serum cortisol concentrations.<sup>16</sup> The aim of this study was to investigate breed variation in plasma concentration of ET-1, plasma renin activity, and serum concentration of cortisol in healthy dogs.

## Materials and Methods

### Animals

Dogs were examined at 5 centers: University of Liège, Belgium; University of Copenhagen, Denmark; National Veterinary School of Alfort, France; University of Helsinki, Finland; and Swedish University of Agricultural Sciences, Sweden. The study was performed as part of the EU-funded LUPA-project,<sup>10</sup> and was approved by an ethical committee in each participating country. Dogs were privately owned, and informed owner consent was obtained. To be included, dogs had to be pure-bred, healthy, and 1-7 years old. Dogs had to have a normal body condition score, and could not be related to each other at parental level. Each center could include 2-5 breeds, and within center, each breed cohort included dogs of 1 sex only, intact males, or females that were spayed or in anestrus, according to LUPA inclusion criteria. Dogs were excluded if any finding indicating systemic or organ-related disease was observed during the clinical examination described below.

### Preparations

To ensure consistent salt intake, owners were instructed to feed their dog only commercial dog food and no treats, 2 weeks before participation in the study. On examination day, all dogs were fasted  $\geq 12$  hours and had no access to water for at least 2 hours before examination.

### Characterization of Health Status

From each dog, a morning urine sample collected by the owner was brought into clinic. Standard urine analysis was performed by

dipstick chemistry test, and refractometer for urine specific gravity. The dog underwent general physical examination including blood pressure measurement by high-definition oscillometry, according to published guidelines.<sup>17</sup> Each dog was assigned a body condition score on a 1-9 points scale.<sup>18</sup> Five-minute ECG recording and echocardiographic examination including 2-dimensional, M-mode, and Doppler echocardiography was performed. The echocardiographic examination was performed from right and left sides, using standardized imaging planes<sup>19</sup> and continuous ECG monitoring. Blood was collected after health examination by venipuncture into 5-mL serum and EDTA tubes. In most dogs, blood sampling was performed between 9 am and 2 pm ( $n = 499$ ), while a few dogs ( $n = 32$ , 6% of total number of dogs) were sampled between 2 and 5 pm. Routine hematology and biochemistry analyses including parameters of liver and kidney function, glucose, and serum electrolyte concentrations were performed. All examinations were performed in unsedated dogs.

### Analyses of ET-1, Renin Activity, and Cortisol

EDTA tubes for analysis of ET-1 concentration and renin activity, and serum tubes for analysis of cortisol concentration, were centrifuged within 30 minutes of sample collection. Plasma and serum were harvested, transferred into plastic cryotubes, frozen and stored at  $-80^{\circ}\text{C}$ . For practical reasons, at 1 center samples were stored at  $-20^{\circ}\text{C}$  for maximum 2 weeks, after which they were transferred frozen to  $-80^{\circ}\text{C}$  and stored for batched analysis. All samples were later transported frozen on dry ice at  $-80^{\circ}\text{C}$  to 2 laboratories, 1 laboratory<sup>a</sup> for analysis of renin activity and ET-1, and another laboratory<sup>b</sup> for analysis of cortisol.

All analyses were performed using commercially available assays validated for dogs,<sup>20-22</sup> according to manufacturers' instructions. Renin activity was measured by radioimmunoassay (RIA) with solid-phase-coated tube separation,<sup>c</sup> ET-1 by ELISA<sup>d</sup> and cortisol by enzyme-linked fluorescent assay (ELFA).<sup>e</sup> All samples were analyzed in duplicate by personnel blinded to dog identity, and mean values were used for data analysis. In-house duplicate coefficients of variation (CVs) were: renin activity 13.8%, ET-1 7.6%, and cortisol 10.5%. Interassay CVs were: renin activity  $< 15\%$ , ET-1  $< 10\%$ , and cortisol  $< 12\%$ . Dynamic ranges were: renin activity 0.17-3.40 ng/mL/h, ET-1 0.39-50 pg/mL, and cortisol 5.5-2,759 nmol/L; and limits of detection were: renin activity 0.010 ng/mL/h, ET-1 0.23 pg/mL, and cortisol 5.5 nmol/L.

### Statistical Analyses

Commercially available software<sup>f</sup> was used for statistical analyses. Data are presented as medians and interquartile ranges (IQR). A value of  $P < .05$  was considered significant, unless otherwise indicated.

The nonparametric Kruskal-Wallis test was used to investigate overall differences among breeds for renin activity, ET-1, and cortisol, respectively. If a significant difference was detected, pair-wise breed comparisons were performed by Mann-Whitney *U*-test with Bonferroni adjustment; significance level  $P < .0014$ .

Due to the uneven breed distribution between centers, breed was highly covariate with center. Kruskal-Wallis test was therefore also used to investigate breed differences within each center for renin activity, ET-1, and cortisol, respectively. At centers including more than 2 breeds, pair-wise breed comparisons were performed by Mann-Whitney *U*-test with Bonferroni adjustment, if an overall significant difference was detected.

Univariate regression analyses were performed to evaluate potential associations between breed, age, body weight, examination center, and renin activity, ET-1, and cortisol, respectively. A

subanalysis of the same variables by univariate regression analysis was performed in Labrador retrievers, because this breed included the largest number of dogs, was represented at 4 of 5 centers and included both female and male dogs. Therefore, sex also was assessed by univariate regression analysis in the Labrador retriever breed.

To compensate for influence of other confounding factors on renin activity, ET-1, and cortisol, multiple regression analysis was performed, including variables that reached  $P < .2$  in the univariate regression analysis of all dogs. Analyses were performed in a reverse stepwise manner,<sup>23</sup> starting with all included variables and removing the variable with highest  $P$ -value until all remaining variables had a  $P$ -value  $< .05$ . All variables were assessed only as main effects; no interaction terms were considered in the model.

Distribution of residuals in the multiple regression analysis was tested for normality using Shapiro-Wilk  $W$  test. The adjusted  $R^2$  is defined as the percentage of the total sum of squares that can be explained by the regression and also considers the degrees of freedom for variables added. Univariate and multiple regression analyses were performed on log-transformed data. No multiple regression analysis was performed in the Labrador retriever cohort due to high covariance between center and sex.

## Results

In total, 531 dogs of 9 breeds were included. Twenty-six examined dogs were excluded due to the following reasons: myxomatous mitral valve disease ( $n = 10$ ), tricuspid dysplasia ( $n = 2$ ), aortic stenosis ( $n = 3$ ), arrhythmia ( $n = 1$ ), hepatopathy ( $n = 1$ ), signs of inflammation on blood panel ( $n = 1$ ), isosthenuria ( $n = 1$ ), underweight ( $n = 1$ ), obesity ( $n = 1$ ), poor condition ( $n = 1$ ), nonfasted at examination ( $n = 1$ ), on medication at examination ( $n = 1$ ), extreme stress during examination ( $n = 1$ ), character of dog (aggression) ( $n = 1$ ). Distribution of breeds and dogs included at different centers is shown in Table 1. Each center examined dogs belonging to 2–4 breeds and some breeds were examined at more than 1 center. One breed, the Labrador retriever, was represented at 4 of 5 centers. Sex distribution was uneven with 413 males and 118 female dogs (Table 1). All males were intact, whereas females were spayed or in anestrus. Median age

( $n = 531$ ) was 3.3 (IQR, 2.6–4.4) years and median body weight ( $n = 490$ ) was 30.0 (IQR, 23.5–36.0) kg.

### ET-1, Renin Activity, and Cortisol

Median ET-1 concentration ( $n = 506$ ) was 1.29 (IQR, 0.97–1.82) pg/mL, median cortisol concentration ( $n = 528$ ) was 46.0 (IQR, 29.0–80.8) nmol/L, and median renin activity ( $n = 528$ ) was 0.73 (IQR, 0.48–1.10) ng/mL/h. The number of missing samples was  $n = 25$  for ET-1,  $n = 3$  for cortisol, and  $n = 3$  for renin activity. Concentrations of ET-1 and cortisol, and renin activity by breed are shown in Fig 1 and Table 2.

**Group-wise Comparisons, All Dogs.** Overall significant breed differences were found for ET-1 and cortisol concentrations, and renin activity ( $P$  for all  $< .0001$ ). Pair-wise breed comparisons showed significant differences for 24 of 36 comparisons (67%) for ET-1, 8 of 36 comparisons (22%) for cortisol, and 7 of 36 comparisons (19%) for renin activity (Table 3). Concentration of ET-1 was highest in Newfoundlands with a median concentration more than 3 times higher than median values in Cavalier King Charles Spaniels (CKCS), Doberman Pinschers and Dachshunds, which had lowest concentrations. Renin activity was highest in Dachshunds with a median value twice the values in Boxers and Newfoundlands, which had the lowest activities. Cortisol concentration was highest in Finnish Lapphunds with a median almost 3 times higher than in Boxers, which had the lowest concentration (Fig 1).

**Group-wise Comparisons, Within Center.** Overall significant breed differences were found at 5 of the 5 centers for ET-1 ( $P \leq .030$ ), at 4 centers for cortisol ( $P \leq .043$ ), and at 2 centers for renin activity ( $P \leq .0002$ ). Pair-wise comparisons showed significant differences in 8 of 16 comparisons of ET-1, 5 of 15 comparisons of cortisol, and 4 of 4 comparisons of renin activity.

**Univariate Regression Analysis, All Dogs.** The univariate regression analysis of all dogs showed association between ET-1 and breed ( $R^2 = 0.39$ ,  $P < .0001$ ), as well as center of examination ( $R^2 = 0.24$ ,  $P < .0001$ ). Cortisol was associated with breed ( $R^2 = 0.08$ ,  $P < .0001$ ) and center of examination ( $R^2 = 0.09$ ,  $P < .0001$ ), and renin activity with breed ( $R^2 = 0.07$ ,  $P < .0001$ ) and center of examination ( $R^2 = 0.02$ ,  $P = .013$ ). ET-1 increased with increasing body weight ( $R^2 = 0.14$ ,  $P < .0001$ ) and renin activity decreased with increasing body weight ( $R^2 = 0.02$ ,  $P = .0009$ ). Age was not associated with any of the 3 examined substances.

**Univariate Regression Analysis, Labrador Retrievers.** The univariate regression analysis of Labrador retrievers showed association between center of examination and cortisol ( $R^2 = 0.19$ ,  $P < .0001$ ) as well as renin activity ( $R^2 = 0.14$ ,  $P = .0003$ ), whereas ET-1 was not associated with center of examination. Sex, age, and bodyweight were not associated with renin activity, ET-1, or cortisol.

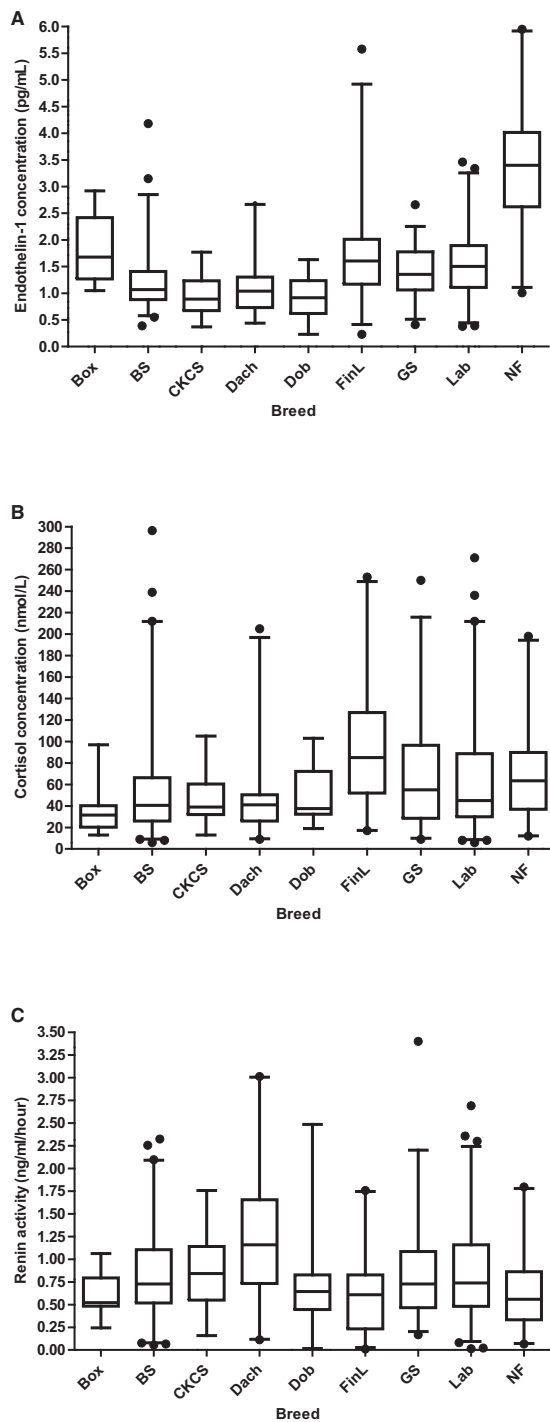
**Multiple Regression Analysis, All Dogs.** In the multiple regression analysis for ET-1, only breed ( $P < .0001$ ) remained significant in the final model with an adjusted

**Table 1.** Distribution of dogs by center of examination, breed, and sex.

	Belgium	Denmark	Finland	France	Sweden	Total
Box					15M	15
BS	95M			25M		121
CKCS					34M	34
Dach			26M		16M	42
Dob				24M		23
FinL			50M			50
GS	16M		60M			76
Lab	6M	45F		29F	46M	126
NF		44F				44
Total	117	89	136	78	111	531

Box, Boxer; BS, Belgian Shepherd; CKCS, Cavalier King Charles Spaniel; Dach, Dachshund; Dob, Doberman pinscher; FinL, Finnish Lapphund; GS, German Shepherd; Lab, Labrador retriever; NF, Newfoundland; M, male; F, female.





**Fig 1.** Boxplots showing distribution of endothelin-1 (A), cortisol (B), and renin activity (C) by breed. The top, bottom, and line within each box correspond to the 75th percentile (top quartile), the 25th percentile (bottom quartile), and the 50th percentile (median), respectively. The whiskers extend from the bottom 2.5th percentile to the top 97.5th percentile. Outliers, represented by black dots, were included in statistical analyses. There was an overall significant breed difference for all 3 substances ( $P < .0001$ ). For information on which breeds that differed significantly, see Table 3. Box, Boxer; BS, Belgian Shepherd; CKCS, Cavalier King Charles Spaniel; Dach, Dachshund; Dob, Doberman pinscher; FinL, Finnish Lapphund; GS, German Shepherd; Lab, Labrador retriever; NF, Newfoundland.

model  $R^2$  of 0.39. For cortisol, multiple regression analysis confirmed an effect of breed ( $P < .0001$ ) and center of examination ( $P < .0001$ ) with an adjusted model  $R^2$  of 0.14. For renin activity, breed ( $P < .0001$ ) and center of examination ( $P = .0002$ ) remained significant in the final model, which had an adjusted model  $R^2$  of 0.11.

## Discussion

Results of this study demonstrate that ET-1 can be included among vasoactive substances that differ between breeds, whereas breed difference was smaller for renin activity. There are breed differences in concentrations of natriuretic peptides and catecholamines in the same healthy dogs.<sup>11,15</sup> Several of these substances are altered in development of cardiac or renal disease and have been investigated as potential biomarkers for disease in dogs.<sup>4-6,8,24</sup> A clinically useful test requires an upper reference limit for healthy dogs, and cut-off values for dogs with subclinical disease or clinical signs of disease.

Endothelin-1 is mainly produced in vascular endothelial cells and acts locally as a potent vasoconstrictor, but it is also produced in organs such as the heart and kidney.<sup>2,25</sup> In dogs, increased concentrations of ET-1 have been found in chronic kidney disease and ET-1 has been suggested a biomarker of hypertension.<sup>6</sup> In one study, plasma concentrations of ET-1 were twice as high in dogs with congestive heart failure compared to controls,<sup>26</sup> and studies indicate that ET-1 could aid in distinguishing between cardiac and noncardiac causes of dyspnea in dogs.<sup>5,8</sup> ET-1 has also been suggested an useful predictor of poor prognosis in dogs with dilated cardiomyopathy.<sup>4</sup> In that study, ET-1 concentrations were 3 times higher in dogs with overt dilated cardiomyopathy compared to controls. Among included breeds in this study, Newfoundlands had the highest median ET-1 concentration with values more than 3 times higher than median values in CKCS, Doberman Pinschers, and Dachshunds. In our previous study of natriuretic peptide concentrations in the same population of dogs, interestingly, Newfoundlands were found to have the highest and Dachshunds the lowest NT-proBNP concentration.<sup>11</sup> ET-1 has been shown to directly induce BNP transcription in cultured ventricular myocytes<sup>27</sup> and ET-1 has also been suggested to be involved in increased synthesis of natriuretic peptides in dogs with congestive heart failure.<sup>28</sup>

In this study, RAAS activation was evaluated by measurement of renin activity. Renin release is stimulated by decrease in arterial blood pressure, decrease in sodium chloride concentration and flow in the nephron, and increase in sympathetic nervous activity.<sup>29</sup> The RAAS is activated in cardiac and renal disease and blockers of different components of RAAS are used therapeutically for both cardiac and renal disease in dogs.<sup>20,30,31</sup> Despite an overall significant breed difference, significant differences were found in less than a fifth of pair-wise comparisons, and within only 2 of the centers. It was primarily one breed which differed from the others in renin activity, namely the Dachshunds

**Table 2.** Median, interquartile range (IQR), minimum (min) and maximum (max) values for endothelin-1 and cortisol concentrations, and renin activity displayed by breed.

Breed	Endothelin-1 (pg/mL)		Cortisol (nmol/L)		Renin Activity (ng/mL/h)	
	Median (IQR)	Min–Max	Median (IQR)	Min–Max	Median (IQR)	Min–Max
Box	1.68 (1.27–2.42)	1.05–2.92	31.5 (20.3–40.3)	13.0–97.0	0.52 (0.48–0.79)	0.24–1.06
BS	1.07 (0.88–1.41)	0.39–4.18	39.0 (26.0–65.0)	6.0–321	0.73 (0.52–1.11)	0.05–2.33
CKCS	0.89 (0.68–1.24)	0.37–1.77	39.0 (32.0–60.5)	13.0–105	0.84 (0.55–1.14)	0.16–1.76
Dach	1.04 (0.73–1.30)	0.44–2.68	41.0 (26.0–50.5)	9.0–205	1.16 (0.73–1.66)	0.11–3.01
Dob	0.92 (0.63–1.24)	0.23–1.63	37.5 (32.3–72.3)	19.0–103	0.64 (0.45–0.83)	0.01–2.49
FinL	1.61 (1.17–2.01)	0.23–5.58	85.0 (52.0–127)	17.0–253	0.61 (0.23–0.83)	0.01–1.76
GS	1.36 (1.06–1.78)	0.41–2.66	55.0 (28.5–96.5)	9.0–250	0.73 (0.47–1.08)	0.17–3.40
Lab	1.51 (1.11–1.90)	0.38–3.46	45.0 (30.0–88.8)	6.0–271	0.74 (0.48–1.16)	0.01–2.69
NF	3.40 (2.62–4.02)	1.01–5.95	63.5 (37.0–89.8)	12.0–198	0.56 (0.33–0.86)	0.07–1.80

Box, Boxer; BS, Belgian Shepherd; CKCS, Cavalier King Charles Spaniel; Dach, Dachshund; Dob, Doberman pinscher; FinL, Finnish Lapphund; GS, German Shepherd; Lab, Labrador retriever; NF, Newfoundland.

**Table 3.** Pair-wise comparisons between breeds in endothelin-1 and cortisol concentrations, and renin activity.

	Box	CKCS	Dach	Dob	FinL	GS	Lab	NF	
BS	*				*	*	*	*	Endothelin-1
					*				Cortisol
			*						Renin activity
Box		*	*	*				*	Endothelin-1
			*		*			*	Cortisol
									Renin activity
CKCS					*	*	*	*	Endothelin-1
					*				Cortisol
									Renin activity
Dach					*	*	*	*	Endothelin-1
					*			*	Cortisol
				*	*	*	*	*	Renin activity
Dob					*	*	*	*	Endothelin-1
					*				Cortisol
									Renin activity
FinL							*	*	Endothelin-1
									Cortisol
									Renin activity
GS								*	Endothelin-1
									Cortisol
									Renin activity
Lab								*	Endothelin-1
									Cortisol
									Renin activity

Box, Boxer; BS, Belgian Shepherd; CKCS, Cavalier King Charles Spaniel; Dach, Dachshund; Dob, Doberman pinscher; FinL, Finnish Lapphund; GS, German Shepherd; Lab, Labrador retriever; NF, Newfoundland.

The asterisks denote significant differences using a Bonferroni corrected *P*-value of <.0014.

which had the highest activity. The same breed had the lowest NT-proBNP concentration of all breeds,<sup>11</sup> and a low median ET-1 concentration. Newfoundlands, on the other hand, had low renin activity and high ET-1 as well as NT-proBNP concentration.<sup>11</sup> Natriuretic peptides are known to suppress RAAS activation in dogs as well as people,<sup>32,33</sup> and ET-1 has been suggested to inhibit renin production, but the interaction between hormonal systems is complex.<sup>34</sup> Taken together, the present results indicate that if ET-1 and natriuretic peptides are high, the renin activity is low and vice versa. Why Dachshund and Newfoundlands had opposite

response patterns is currently unknown. Further study into the interplay between these substances in healthy dogs and in dogs with cardiac and renal disease is warranted to optimize their use as biomarkers of disease.

Dogs in the LUPA project all underwent a thorough standardized health examination. Although attempts were made to stress dogs as little as possible, in a sub-population of dogs examined at one center, we found substantial increases in urine epinephrine and norepinephrine postexamination compared to pre-examination in home environment, indicating that dogs experienced stress or excitement during travel and

examination at the clinic.<sup>15</sup> We also found breed differences in the 2 catecholamines, with lower concentrations in Labrador retrievers compared to CKCS and Dachshunds.<sup>15</sup> In this study, we analyzed cortisol concentration in serum samples taken from all dogs after the clinical examination. The observation that breed differences were found within 4 of the 5 centers indicates that differences in handling of the dogs between centers could not be the sole explanation. In contrast to the catecholamines, no breed differences were shown between CKCS, Dachshunds, and Labrador retrievers. Instead, cortisol concentration was highest in Finnish Lapphunds with median concentration almost 3 times higher than median concentration in Boxers, which had the lowest concentration. However, although statistically significant, the actual breed difference in cortisol concentration was comparatively small and variation within breeds substantial. The individual stress response might vary and other factors than breed might be more relevant for an individual dog in the clinical situation.

Other physiologic factors, which might influence concentration of vasoactive peptides, include sex and age.<sup>35,36</sup> Due to the uneven sex distribution among centers as well as breeds, sex was not included in the univariate or multiple regression analyses of all dogs, but was instead assessed in the univariate regression analysis of the Labrador retriever cohort. No association was found between sex and ET-1 concentration, cortisol concentration, or renin activity. This study was, however, not designed to evaluate sex differences and no multiple regression analysis was performed in the Labrador retriever cohort, due to the high covariance between center and sex. Age was not associated with any of the 3 investigated substances, neither including all dogs nor in the Labrador retriever cohort. This was not surprising, as the study only included young adult to middle-aged dogs and was not designed to evaluate age differences. Age and sex differences have been shown for cortisol and components of RAAS in dogs.<sup>37–39</sup> Age and sex differences have been shown for ET-1 in people,<sup>40,41</sup> whereas scarce information is available for dogs. Additional study into the effect of sex and age on investigated variables in dogs is warranted.

### Study Limitations

Breed differences in ET-1 concentration were highly significant within all 5 centers. However, the study only included 9 breeds and cannot be considered representative of the entire dog population. Because of the uneven sex representation and narrow age span of included dogs, results should not be interpreted as reference values. To establish breed-specific reference values, studies evaluating additional breeds with an even sex and age representation would have to be performed. Breed differences were found at 2 centers for renin activity and at 4 centers for cortisol concentration. For the latter 2 substances, the different number of individuals of each breed and the uneven breed distribution among centers

could have played a role because the variation was small compared to ET-1. Furthermore, within-dog variation in the substances have not been tested, and could have affected the results.

Sample handling was standardized, but samples were kept frozen at  $-20^{\circ}\text{C}$  for a short time at 1 of the 5 centers. Stability has been shown at the freezing temperatures used for cortisol in dogs and for plasma renin activity in people.<sup>42,43</sup> Stability of ET-1 has not been investigated in dogs, but in one human study, ET-1 was stable at  $-80^{\circ}\text{C}$ , but showed degradation when stored at  $-30^{\circ}\text{C}$  for 3 weeks.<sup>44</sup> Center of examination did not affect ET-1 concentration in the multiple regression analysis of all dogs or in the univariate regression analysis of the Labrador retriever cohort, where this center was included. Hence, it is unlikely that sample handling had any major effect on the results.

The interassay variabilities in this study were good ( $<10\%$ ) to acceptable ( $<15\%$ ), and should therefore not have had any major effect on the results. Another factor which could affect results is time-to-time variation. In dogs, circadian variation has been shown for serum cortisol<sup>45</sup> and plasma renin activity,<sup>46</sup> but has not been investigated for plasma ET-1. In a small human study, time-to-time variation in ET-1 was found.<sup>47</sup> To account for circadian variation, blood sampling was performed between 0900 and 1400 hours in this study. However, for practical reasons 6% of the dogs were instead sampled between 1400 and 1700 hours, which could have had a minor effect on the results. Further study into time-to-time variation in ET-1 in dogs is warranted.

In order to avoid excessive salt intake, dogs were fed only commercial dog foods and no treats for 2 weeks preceding examination. However, as salt content might vary between food brands, salt intake between dogs could not be standardized which might have affected plasma renin activity. Dogs had no access to water for at least 2 hours before examination in order not to affect urine density measurements and assessment of renal function. According to newly published human recommendations,<sup>48</sup> water should be allowed during the fasting period for most plasma/serum blood tests and the ingested volume of water should mirror the usual daily ingested water volume of each individual. However, urine density as well as hematocrit was within reference range for all participating dogs and water restriction should therefore not have had any major effect on the results.

### Conclusion

Breed variation was considerable in circulating ET-1, but less prominent for circulating cortisol and renin activity in healthy dogs. Evidence for breed variation in yet another vasoactive peptide highlights the need for deeper understanding of their role in health and disease. Furthermore, breed variation might be important to take into consideration when interpreting test results in clinical studies.

## Footnotes

- <sup>a</sup> Laboratoire de Physiologie, Faculté de Médecine, Bruxelles, Belgium  
<sup>b</sup> Laboratoire Vebio, Arcueil, France  
<sup>c</sup> Plasma renin activity, RIA, Diasorin, Stillwater, MN  
<sup>d</sup> ET-1, ELISA, IBL-27165, Aramachi, Takasaki-Shi, Gunma, Japan  
<sup>e</sup> Cortisol, ELFA, VIDAS, Biomérieux SA, Lyon, France  
<sup>f</sup> JMP Pro, version 11.0.0, SAS Institute Inc, Cary, NC

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**Conflict of Interest Declaration:** Authors disclose no conflict of interest.

**Off-label Antimicrobial Declaration:** Authors declare no off-label use of antimicrobials.

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